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(54) Immunoassay for the detection of amphetamines and derivatives thereof

(57) The present invention provides an immunoassay method for the highly sensitive detection of amphetamines, methamphetamines, and methylenedioxy designer amphetamines in urine samples. Commercial-

ly available reagents for the determination of amphetamines and methamphetamines are used with a calibrator comprising a known amount of a substance selected from the group consisting of methylenedioxy designer amphetamines.

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Description

BACKGROUND OF THE INVENTION

5 [0001] This invention relates generally to the field of measuring an analyte in a liquid medium. More specifically, it relates to an assay for the measurement of a drug of abuse in a biological sample. In particular, the invention relates to a highly sensitive immunoassay method for the detection of amphetamines, methamphetamines, structurally related drugs such as 3,4-methylenedioxymethamphetamine (MDMA) and metabolites of these drugs in biological samples.

10 [0002] The amphetamine analogues of methylenedioxyphenylalkylamine are a series of compounds referred to as "designer" amphetamines. As represented in Figure 1, these psychotropic drugs are ring-substituted derivatives chemically related to mescaline. They include methylenedioxyamphetamine (MDA), methylenedioxyamphetamine (MDMA, also known as Ecstasy), methylenedioxyethylamphetamine (MDEA), N-methylbenzodioxazolybutanamine (MBDB) and benzodioxazol-5'-yl-2-butanamine (BDB), the most common of these being MDMA.

15 [0003] MDA has been shown to be the metabolite of both MDMA and MDEA. Several animal studies have shown that MDMA is metabolized by N-demethylation, deamination, O-methylation and O-conjugation to glucuronide and/or sulfate metabolites. Detected in urine are the parent drug (MDMA), 3,4-methylenedioxyamphetamine (MDA), 4-hydroxy-3-methoxymethamphetamine (HMMA), 3-hydroxy-4-methoxymethamphetamine, 4-hydroxy-3-methoxyphenylacetone, 3,4-methylenedioxyphenylacetone and 3,4-dihydroxyphenylacetone. Most of these metabolites are also present in the blood.

20 [0004] Urine and blood are the most commonly studied biological matrices for MDMA, MDA, MDEA and MBDB and are well documented in the literature. Determination of these designer drugs in other biological specimens such as saliva, sweat and hair has been reported more recently. The parent drug is detected in higher concentrations than its metabolites in these matrices.

25 [0005] The abuse of these designer amphetamines is increasing throughout the world, and their detection by screening methods is becoming a more important issue. Zhao, H. et al., *J. Anal. Toxicology*, (2000) found 71 % of urine samples from rave party attendees contained MDMA or MDA alone or in combination with amphetamine or other designer amphetamines such as MDEA. Presently there are no commercial immunoassays designed specifically for the detection of these substances, and their detection therefore depends on the relative cross-reactivities they exhibit in the amphetamine or methamphetamine screening method used. In general, the cross-reactivity of the commercially available amphetamine and methamphetamine assays toward many of these compounds is low which suggests the possibility that some positive samples may go undetected.

30 [0006] In testing for drugs of abuse, immunoassays, particularly competitive binding immunoassays, have proven to be especially advantageous. In competitive binding immunoassays, an analyte in a biological sample competes with a labeled reagent, or analyte analog, or tracer, for a limited number of receptor binding sites on antibodies specific for the analyte and analyte analog. Enzymes such as β -galactosidase and peroxidase, fluorescent molecules such as fluorescein compounds, radioactive compounds such as ^{125}I , and microparticles are common labeling substances used as tracers. The concentration of analyte in the sample determines the amount of analyte analog which will bind to the antibody. The amount of analyte analog that will bind is inversely proportional to the concentration of analyte in the sample, because the analyte and the analyte analog each bind to the antibody in proportion to their respective concentrations. The amount of free or bound analyte analog can then be determined by methods appropriate to the particular label being used.

35 [0007] Gas chromatography/mass spectrometry (GC/MS) is highly specific and has been described for the simultaneous detection of MDMA, MDA, amphetamine, methamphetamine, MDEA and their metabolites. GC/MS analysis is usually required for confirmation and verification of the results of an immunological assay or a suspected diagnosis. In this technique, MDMA or designer drugs are extracted in solid phase, then derivatized and analyzed via GC/MS.

40 [0008] In U.S. Patent No. 5,501,987 issued March 26, 1996, Ordonez et al. describe a dual analyte immunoassay for the determination of amphetamine and methamphetamine using a single labeled binding partner capable of cross reacting at differing sensitivities to antibodies derived from conjugate derivatives of amphetamine and methamphetamine. Calibrators used are prepared by adding d-amphetamine to drug-free, normal human urine.

SUMMARY OF THE INVENTION

45 [0009] Quite surprisingly, it has been discovered that a highly specific immunoassay method for the detection of amphetamines, methamphetamines, structurally related drugs such as 3,4-methylenedioxyamphetamine (MDMA) and their metabolites in urine samples can be achieved by the use of a calibrator comprising a substance selected from the group consisting of methylenedioxy designer amphetamines in drug free, normal human urine and an antibody having specificity for amphetamine or methamphetamine and cross-reactivity with amphetamine analogues of methylenedioxyphenylalkylamine.

[0010] In the method of the invention, a sample suspected of containing amphetamine, methamphetamine or a structurally related drug is combined with an antibody having specificity for amphetamine or methamphetamine and a labeled binding partner which can interact with the combination of antibody and its corresponding analyte so as to detect the presence of the analytes at selected cutoff levels either alone or in combination. The particular antibody or antibodies used must have cross-reactivity with amphetamine analogues of methylenedioxyphenylalkylamine. This invention can be used with any type of immunoassay format, e.g., turbidometric agglutination assay, radioimmunoassay, enzyme immunoassay, or fluorescent polarization immunoassay. Especially preferred is the use of the present invention with agglutination formats susceptible to an instrumental method for the measurement of the changes brought about by the agglutination reaction. Both manual as well as automated apparatus testing may be suitably employed for such agglutination analysis. Typically, automated instrumentation will operate utilizing a multiplicity of reagent containers or reservoirs from which will be pipetted the appropriate amount of each reagent for addition to the sample. For immunoassays such as the subject agglutination assay, this will usually involve at least two such containers; typically, one for an antibody reagent and the other for the microparticles bound with the corresponding ligand. Additional containers or reservoirs may be present in some instruments containing diluent, buffers or other additives for appropriate treatment of the sample.

[0011] The clinical analyzer pipettes the onboard reagents and samples into one cuvette where the competitive agglomeration reaction occurs and measurement of the turbidity is made. For example, using the HITACHI 917 analyzer (Roche Diagnostics) and the ABUSCREEN® OnLine Amphetamines reagent kit (Roche Diagnostics, Cat. No. 1985965), urine sample is pipetted with sample diluent into the cuvette, followed immediately by the appropriate amount of antibody reagent and mixing. An initial spectrophotometer reading is taken. Then the appropriate quantity of microparticle reagent is transferred to the cuvette and the reaction mixed. After a brief incubation, a final turbidity measurement is made. The overall change in turbidity (absorbance) in the reaction is compared to a calibration curve and results reported in ng/ml.

[0012] The present invention also encompasses a reagent test kit which comprises, in packaged combination, an antibody specific for amphetamine, an antibody specific for methamphetamine, a complex comprising a ligand of amphetamine or an amphetamine derivative coupled to a labeling moiety, and a calibrator comprising a known amount of a substance selected from the group consisting of methylenedioxy designer amphetamines. Such a test kit provides reagents for an assay with enhanced clinical sensitivity for MDMA and structurally-related compounds.

BRIEF DESCRIPTION OF THE DRAWINGS

[0013]

Figure 1 shows the structures of amphetamine, methamphetamine and 3,4-methylenedioxy designer drugs.

Figure 2 is a dose response curve generated using the assay of the present invention comprising an antibody specific for amphetamine, an antibody specific for methamphetamine and calibrators comprising known amounts of MDMA.

Figure 3 is a dose response curve generated using the assay of the present invention comprising an antibody specific for amphetamine, an antibody specific for methamphetamine and calibrators comprising known amounts of MDA.

DETAILED DESCRIPTION OF THE INVENTION

[0014] Commercial immunoassay kits for determination of MDMA are currently not available. The only way to determine MDMA via immunoassay is to use reagents or a reagent kit for determining amphetamine or methamphetamine comprising an amphetamine antibody and methamphetamine antibody having high cross-reactivity with MDMA and using amphetamine or methamphetamine as a calibrator. In the present invention, a substance selected from the group consisting of methylenedioxy designer amphetamines is used to calibrate a commercially available assay using antibodies for amphetamine and methamphetamine. The use of a methylenedioxy designer amphetamine calibrator significantly increased the clinical sensitivity for MDMA, MBDB, MDA, MDE, and BDB without significant increase for medications such as β -hydroxyphenylamines, e.g., ephedrine, pseudoephedrine, phentamine, tyramine and phenylpropanolamine (PPA).

[0015] Abbreviations used:

AMP amphetamine

BDB	(±)-(3,4-methylenedioxyphenyl)-2-butanamine
HMMA	4-hydroxy-3-methoxymethamphetamine
5 MAMP	methamphetamine
MBDB	(±)-N-methyl-1-(3,4-methylenedioxyphenyl)-2-butanamine
MDA	(±)-3,4-methylenedioxyamphetamine
10 MDEA	(±)-3,4-methylenedioxyethylamphetamine
MDMA	(±)-3,4-methylenedioxymethamphetamine
15 NT	not tested

PPA phenylpropanolamine

[0016] Cross-reactivities of currently marketed assays for MDMA and MDA, according to published literature, as well as cross-reactivities using the method of the present invention are listed in the table below.

[0017] By "methylenedioxy designer amphetamines" is meant the group of amphetamine analogues of methylenedioxyphenylalkylamine including methylenedioxyamphetamine (MDA), methylenedioxymethamphetamine (MDMA, Ecstasy), methylenedioxyethylamphetamine (MDEA), N-methylbenzodioxazolybutanamine (MBDB) and benzodioxazol-5'-yl-2-butanamine (BDB).

Compound	Roche Hitachi AMP 500 ng/ml	Roche Integra AMPSC 500 ng/ ml	Abbott TDX AMP/ methAMP 1000 ng/ml	CEDIA DAU AMP 1000 ng/ml	Roche Hitachi AMP/MDMA 300 ng/ml
MDMA	36%	79%	97%	69%	100.0%
MDA	35.5%	40%	148%	1.9%	25.0%
MDEA	NT	NT	42.7%	2.4%	15.0%
MBDB	NT	NT	(+)	NT	70.0%
BDB	NT	NT	(+)	NT	4.7%
d-AMP	100%	100%	100%	101%	97.0%
1-AMP	6%	4.2%	56.9%	3.0%	3.8%
d-MAMP	82.2%	12%	97.8%	100%	300.0%
1-MAMP	0.8%	12%	7.2%	12%	18%
dl-ephedrine	<0.1 %	<0.1 %	NT	0.4 %	<0.3 %
1-PPA	1.5%	1.1%	NT	NT	0.6%
40 HMMA	NT	NT	NT	NT	<0.3 %

[0018] Cutoff levels, which are indicated in the above chart for each method, are the concentration of drugs in the sample required for the test to determine a positive result.

Example 1. Preparation of antibody reagent

[0019] A first reagent was prepared according to the directions accompanying the ABUSCREEN® OnLine HS Amphetamine/MDMA reagent kit (Roche Diagnostics, Cat. No. 1986619). The reagent contained amphetamine and methamphetamine monoclonal antibodies (mouse) in a buffer with bovine serum albumin and a preservative.

Example 2. Preparation of microparticle reagent

[0020] A second reagent was prepared according to the directions accompanying the ABUSCREEN® OnLine HS Amphetamine/MDMA reagent kit (Roche Diagnostics, Cat. No. 1986619). The reagent contained an amphetamine derivative conjugated to latex microparticles in a buffer with a preservative.

Example 3. Preparation of MDMA calibrator

[0021] Calibrators were prepared according to the directions accompanying the ABUSCREEN® OnLine Preciset® MDMA calibrators (Roche Diagnostics, Cat. No. 4745556). The calibrator solutions contained 3,4-methylenedioxymethamphetamine in human urine with a preservative. Final concentrations of the calibrators were 0, 150, 300 and 600 ng/ml.

Example 4. Assay using MDMA calibrator

[0022] Calibrators or reference samples prepared according to Example 3 were assayed according to the directions accompanying the OnLine reagent kit using an HITACHI 917 analyzer (Roche Diagnostics) and a cutoff value of 300 ng/ml. Parameters used were 10 µl sample, 160 µl antibody reagent and 90 µl microparticle reagent. The reaction was run monochromatically (505 nm) in the endpoint (read point 19-33). The dose response curve is shown in Figure 2, with the change in absorbance at 505 nm plotted on the y-axis and MDMA concentration plotted on the x-axis.

Example 5. Assay using MDA calibrator

[0023] Calibrators or reference samples prepared as described in Example 3 except using MDA in place of MDMA were assayed according to the directions accompanying the OnLine reagent kit using an HITACHI 717 analyzer (Roche Diagnostics). Parameters used were 15 µl sample, 170 µl antibody reagent and 80 µl microparticle reagent. The reaction was run monochromatically (505 nm) in the endpoint (27-50). The dose response curve obtained is shown in Figure 3, with the change in absorbance at 505 nm plotted on the y-axis and MDA concentration plotted on the x-axis.

[0024] Cross-reactivities observed when MDA was used as a calibrator were as follows:

Compound	Roche Hitachi AMP/MDMA 300 ng/ml
MDMA	81.0%
MDA	100.0%
d-AMP	319.0%
1-AMP	15.2%
d-MAMP	269.0 %
1-MAMP	24.7%
dl-ephedrine	0.34 %
1-PPA	6.2%

Example 6. Assay of urine samples

[0025] Urine specimens suspected of containing amphetamine, methamphetamine or methylenedioxy designer amphetamines were treated according to the procedure described in Example 4 using MDMA calibrators at levels of 0, 150, 300 and 600 ng/ml. Results were obtained by comparing the change in absorbance at 505 nm for an unknown sample with that obtained with the calibrators of known concentration. Results obtained on 72 urine specimens had 100 % agreement with a reference chromatographic method for the detection of designer amphetamines.

Claims

1. A method for determining an analyte selected from the group consisting of amphetamine, methamphetamine, and methylenedioxy designer amphetamines in a biological sample comprising the steps of:

a. combining a sample suspected of containing said analyte with a first antibody specific for amphetamine and having cross-reactivity with amphetamine analogues of methylenedioxyphenylalkylamine, a second antibody specific for methamphetamine and having cross-reactivity with amphetamine analogues of methylenedioxyphenylalkylamine, and a complex comprising a ligand of said analyte coupled to a labeling moiety,

b. measuring the presence or amount of said complex which remains bound or unbound to said antibodies as a result of competitive displacement by said analyte, and

c. comparing the presence or amount of said complex measured in step (b) with the presence or amount of a

similar complex measured in a reference sample containing a known amount of a substance selected from the group consisting of methylenedioxy designer amphetamines, said reference sample being treated according to steps (a) and (b).

- 5 2. The method of claim 1, wherein said sample is urine.
3. The method of claim 2, wherein said substance is MDMA.
- 10 4. A method for determining an analyte selected from the group consisting of amphetamine, methamphetamine, and methylenedioxy designer amphetamines in a biological sample comprising the steps of:
 - 15 a. combining a sample suspected of containing said analyte with an antibody selected from the group consisting of antibodies specific for amphetamine and having cross-reactivity with amphetamine analogues of methylenedioxyphenylalkylamine and antibodies specific for methamphetamine and having cross-reactivity with amphetamine analogues of methylenedioxyphenylalkylamine, and further with a complex comprising a ligand of said analyte coupled to a labeling moiety,
 - 20 b. measuring the presence or amount of said complex which remains bound or unbound to said antibody as a result of competitive displacement by said analyte, and
 - 25 c. comparing the presence or amount of said complex measured in step (b) with the presence or amount of a similar complex measured in a reference sample containing a known amount of a substance selected from the group consisting of methylenedioxy designer amphetamines, said reference sample being treated according to steps (a) and (b).
5. The method of claim 4, wherein said sample is urine.
6. The method of claim 5, wherein said substance is MDMA.
- 30 7. A kit for conducting an assay for the determination of an analyte selected from the group consisting of amphetamine, methamphetamine, and methylenedioxy designer amphetamines in a biological sample comprising in packaged combination:
 - 35 a. a first antibody specific for amphetamine and having cross-reactivity with amphetamine analogues of methylenedioxyphenylalkylamine,
 - b. a second antibody specific for methamphetamine and having cross-reactivity with amphetamine analogues of methylenedioxyphenylalkylamine,
 - 40 c. a complex comprising a ligand of amphetamine or an amphetamine derivative coupled to a labeling moiety, and
 - d. a calibrator comprising a known amount of a substance selected from the group consisting of methylenedioxy designer amphetamines.
 - 45
8. The kit of claim 7, wherein said sample is urine.
9. The method of claim 8, wherein said substance is MDMA.
- 50 10. A kit for conducting an assay for the determination of an analyte selected from the group consisting of amphetamine, methamphetamine, and methylenedioxy designer amphetamines in a biological sample comprising in packaged combination:
 - 55 a. an antibody selected from the group consisting of antibodies specific for amphetamine and having cross-reactivity with amphetamine analogues of methylenedioxyphenylalkylamine and antibodies specific for methamphetamine and having cross-reactivity with amphetamine analogues of methylenedioxyphenylalkylamine,
 - b. a complex comprising a ligand of amphetamine or an amphetamine derivative coupled to a labeling moiety,

and

c. a calibrator comprising a known amount of a substance selected from the group consisting of methylenedioxy designer amphetamines.

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11. The kit of claim 10, wherein said sample is urine.

12. The method of claim 11, wherein said substance is MDMA.

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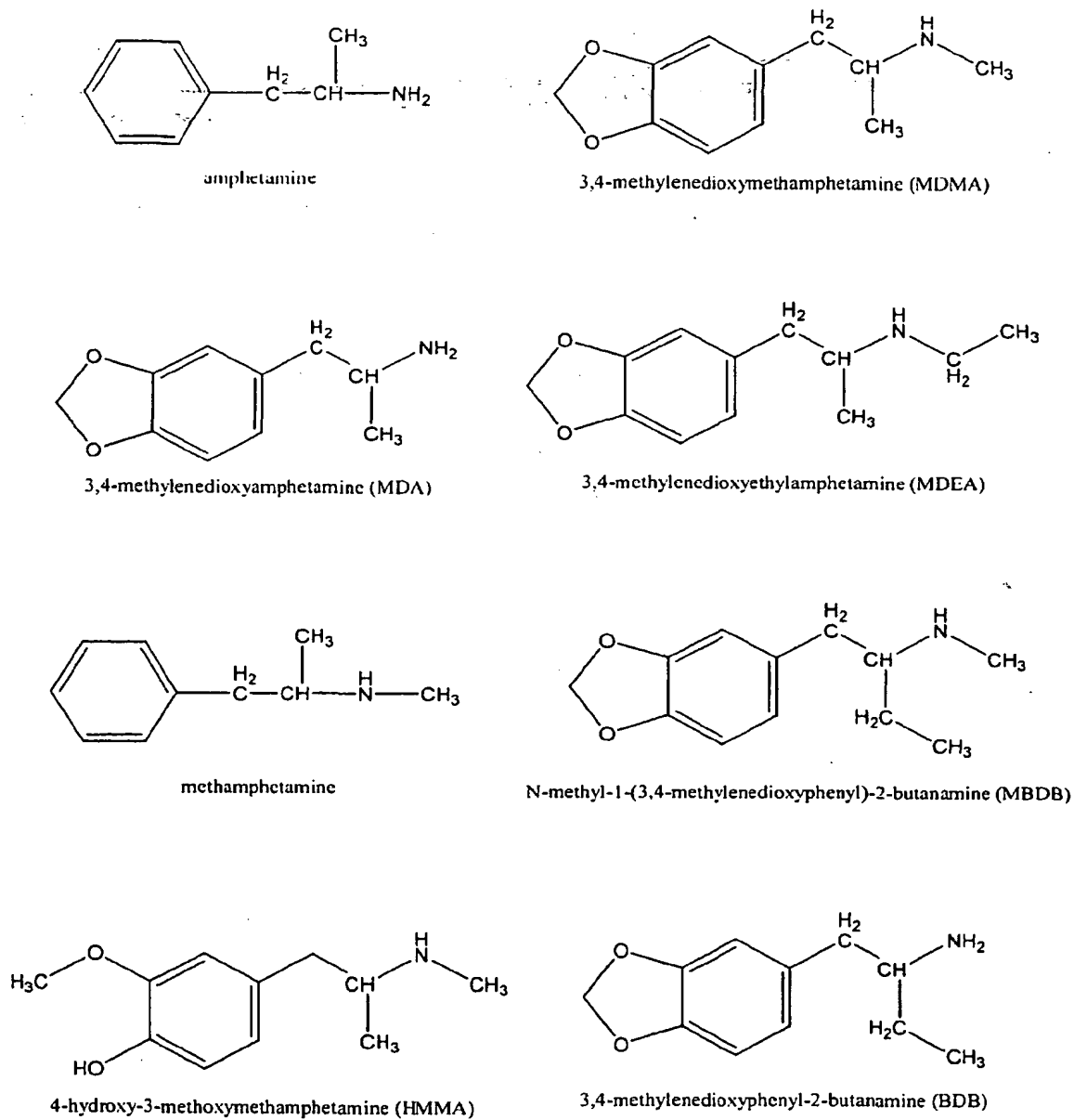


Figure 1

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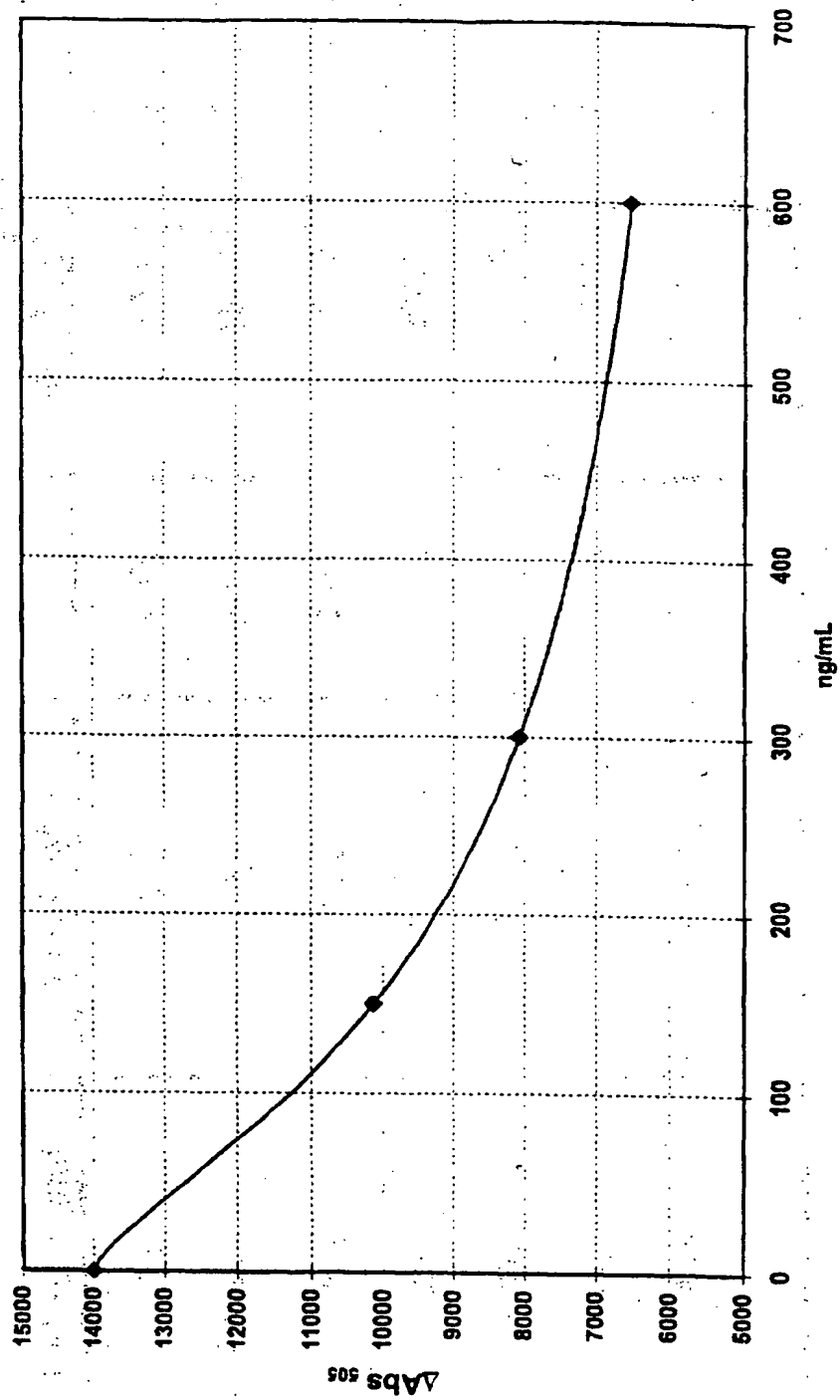
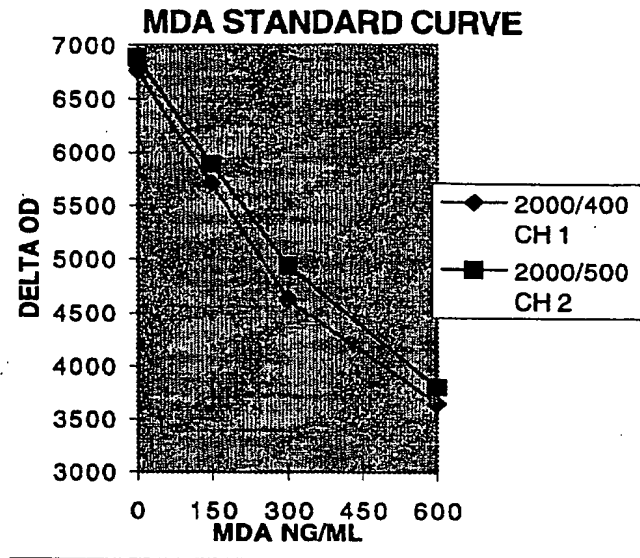


Figure 2



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Figure 3

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EUROPEAN SEARCH REPORT

Application Number
EP 01 11 5342

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The present search report has been drawn up for all claims			
Place of search MUNICH		Date of completion of the search 4 June 2002	Examiner STEINHEIMER, K
CATEGORY OF CITED DOCUMENTS X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	

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EUROPEAN SEARCH REPORT

Application Number
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Place of search MUNICH		Date of completion of the search 4 June 2002	Examiner STEINHEIMER, K
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

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**ANNEX TO THE EUROPEAN SEARCH REPORT
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